Molecular Biology of the Cell
Vol. 18, 1953–1963, May 2007

A Conditional Yeast E1 Mutant Blocks the Ubiquitin–Proteasome Pathway and Reveals a Role for Ubiquitin Conjugates in Targeting Rad23 to the Proteasome

Nazli Ghaboosi and Raymond J. Deshaies

Howard Hughes Medical Institute, Division of Biology, California Institute of Technology, Pasadena, CA 91125

Submitted October 30, 2006; Revised February 20, 2007; Accepted March 1, 2007
Monitoring Editor: William Tansey

E1 ubiquitin activating enzyme catalyzes the initial step in all ubiquitin-dependent processes. We report the isolation of *uba1-204*, a temperature-sensitive allele of the essential *Saccharomyces cerevisiae* E1 gene, *UBA1*. *Uba1-204* cells exhibit dramatic inhibition of the ubiquitin–proteasome system, resulting in rapid depletion of cellular ubiquitin conjugates and stabilization of multiple substrates. We have employed the tight phenotype of this mutant to investigate the role ubiquitin conjugates play in the dynamic interaction of the UbL/UBA adaptor proteins Rad23 and Dsk2 with the proteasome. Although proteasomes purified from mutant cells are intact and proteolytically active, they are depleted of ubiquitin conjugates, Rad23, and Dsk2. Binding of Rad23 to these proteasomes in vitro is enhanced by addition of either free or substrate-linked ubiquitin chains. Moreover, association of Rad23 with proteasomes in mutant and wild-type cells is improved upon stabilizing ubiquitin conjugates with proteasome inhibitor. We propose that recognition of polyubiquitin chains by Rad23 promotes its shuttling to the proteasome in vivo.

INTRODUCTION

Activation of ubiquitin by ubiquitin-activating enzyme (E1) is the requisite first step in all ubiquitin-dependent pathways, including regulated proteolysis. The process begins with an ATP-dependent reaction in which the ubiquitin moiety forms a high-energy thioester bond with the active site cysteine of E1. E1 can then transfer the activated ubiquitin to a conjugating enzyme (E2), which acts alone or in conjunction with a ubiquitin ligase (E3) to covalently link ubiquitin to lysine residues on specific target proteins (Haas and Siepmann, 1997). Through successive ligation reactions, a polyubiquitin chain can form on the substrate and serve as a signal for targeting to the multisubunit 26S proteasome. Composed of a cylindrical 20S proteolytic core complex capped at one or both ends by 19S regulatory complexes, the proteasome deubiquitinates and unfolds substrates before translocating them into its core for proteolysis (Amerik and Hochstrasser, 2004; Pickart and Cohen, 2004).

The existence of mammalian cell lines that carry temperature-sensitive alleles of E1 has been of great importance to the study of the functions of the ubiquitin system in animal cells (Ciechanover *et al*., 1984; Finley *et al*., 1984; Kulka *et al*., 1988; Salvat *et al*., 2000). Ironically, no tight, rapid-acting conditional mutation has been described for the budding yeast *UBA1* gene that encodes E1 despite the availability of sophisticated molecular genetic techniques in this organism. Although a temperature-sensitive allele of *UBA1* exists, this allele results in only moderate stabilization of tested substrates and possible defects in ubiquitination were not examined (McGrath, 1991; Gandre and Kahana, 2002). A second allele resulting from a transposon insertion upstream of the *UBA1* coding sequence reduced wild-type Uba1 protein function, causing inefficient degradation of some proteins (Swanson and Hochstrasser, 2000). Additional alleles were later encountered as suppressor mutations in various indirect genetic screens, and they also achieved only partial inactivation of the pathway (Cheng *et al*., 2002; Shimada *et al*., 2002). Whereas these hypomorphic alleles have proven useful in confirming the ubiquitin dependence of the turnover of specific proteins, they highlight the potential value of a stronger conditional allele with broad utility in exploring the entire ubiquitin conjugation pathway. Among the many issues that could be addressed with a tight and rapid-acting temperature-sensitive mutation in *UBA1* is the question of how ubiquitin conjugates contribute to the proteasomal targeting of substrates destined for proteolysis.

Although the enzymatic cascade responsible for polyubiquitination provides strict control of substrate tagging, the pathway was originally thought to culminate in a stochastic interaction between polyubiquitin chains and the proteasome. On the contrary, recent work has shown that a diverse set of ubiquitin-binding receptors regulate the targeting of ubiquitinated proteins to the proteasome in a substrate-specific manner (Wilkinson *et al*., 2001; Chen and Madura, 2002; Elsasser *et al*., 2004; Verma *et al*., 2004; for review, see Elsasser and Finley, 2005; Hicke *et al*., 2005). The first polyubiquitin-binding receptor identified was Rpn10, a subunit of the 19S regulatory complex that contains a ubiquitin-interacting motif (Deveraux *et al*., 1994). However, cells lacking Rpn10 exhibit only mild phenotypes, indicating that addi-

This article was published online ahead of print in *MBC in Press* (http://www.molbiolcell.org/cgi/doi/10.1091/mbc.E06–10–0965) on March 14, 2007.

Address correspondence to: Raymond J. Deshaies (deshaies@caltech.edu).

tional ubiquitin chain recognition mechanisms must exist (van Nocker *et al*., 1996). A second class of polyubiquitin-binding proteins, referred to as ubiquitin-like/ubiquitin-associated (UbL/UBA) proteins, is exemplified in yeast by the nucleotide excision repair protein Rad23 (Watkins *et al*., 1993) and the spindle pole duplication factor Dsk2 (Biggins *et al*., 1996). UbL/UBA proteins specifically recognize polyubiquitin chains via C-terminal UBA domains (Bertolaet *et al*., 2001b; Wilkinson *et al*., 2001; Hofmann and Bucher, 1996; Rao and Sastry, 2002; Chen and Madura, 2002; Raasi and Pickart, 2003). For example, Rad23 has two UBA domains that interact with polyubiquitinated proteins in a linkage-specific and chain length-dependent manner, preferentially binding to the K48-linked ubiquitin chains that serve as a degradation signal (Raasi and Pickart, 2003). In addition, these proteins interact with the proteasome via their N-terminal UbL domain (Schauber *et al*., 1998; Elsasser *et al*., 2002; Saeki *et al*., 2002b; Walters *et al*., 2002).

Integrating these two recognition motifs, UbL/UBA proteins, are proposed to function as receptors that link polyubiquitinated proteins to the proteasome. Several lines of evidence support this receptor hypothesis. In *rad23* and *dsk2* mutant cells, substrates are ubiquitinated but not degraded, consistent with a role in substrate delivery (Lambertson *et al*., 1999; Wilkinson *et al*., 2001; Chen and Madura, 2002; Funakoshi *et al*., 2002; Rao and Sastry, 2002; Saeki *et al*., 2002a). Moreover, both the UbL and UBA domains of Rad23 are essential for its role in promoting proteolysis (Bertolaet *et al*., 2001b; Lambertson *et al*., 2003; Rao and Sastry, 2002). Most recently, it was shown that Rad23 and Rpn10 can directly promote proteasome binding and degradation of a ubiquitinated substrate in vitro, thereby establishing a direct receptor function for these proteins (Verma *et al*., 2004).

UbL/UBA proteins are emerging as central players in selective proteolysis, and their interactions with numerous other ubiquitin–proteasome system (UPS) elements suggest a complex regulatory scheme, which remains poorly understood. For example, there are reports that UbL/UBA proteins may regulate substrate ubiquitination as well as proteasomal targeting. The UbL domain of Rad23 binds the ubiquitin ligase Ufd2, which cooperates with the chaperone-like AAA ATPase Cdc48 and its cofactors to ubiquitinate specific substrates (Richly *et al*., 2005; Kim *et al*., 2004; Schuberth *et al*., 2004). Moreover, when overexpressed, Rad23 and Dsk2 are capable of inhibiting proteolysis by binding a polyubiquitin chain on a substrate and inhibiting the ligation of additional ubiquitin molecules (Kleijnen *et al*., 2000; Ortolan *et al*., 2000). Even the stoichiometry of receptor–ubiquitin chain interactions is potentially complex given that Rad23 can form homodimers as well as heterodimers with the UbL/UBA protein Ddi1 (Bertolaet *et al*., 2001a,b; Kang *et al*., 2006) and that one polyubiquitin chain may simultaneously capture two receptor molecules (Kang *et al*., 2006; Lowe *et al*., 2006). Finally, intramolecular interactions between UbL and UBA domains may function in regulating UbL/UBA protein activity (Ryu *et al*., 2003; Walters *et al*., 2003; Raasi *et al*., 2004).

In this study, we isolate and characterize a novel allele of the yeast *UBA1* gene, *uba1-204*, in which ubiquitin molecules are not efficiently assembled into chains or conjugated onto substrates. We use the resulting ubiquitin chain-depleted cellular environment to investigate the nature of ubiquitin-dependent proteasomal targeting.

MATERIALS AND METHODS

Yeast Strain Construction

To create the wild-type strain used in all experiments, a deletion of the *UBA1* gene was generated by a gene disruption technique described previously (Guthrie and Fink; 1991). *UBA1* was replaced by *Kanmx*, which was amplified by polymerase chain reaction (PCR) by using oligonucleotides (oligos) NG34 (5′-AAAAAGTAAGATTAGTAGCAAAGCAAAGAACATATAACTATAGC-TTCGACGTACGCTGCAGGTCGAC-3′) and NG37 (5′-GGTTATAACGCA-TAGTGAACAAATGATGAGGTCTTGTAACTCGATTGCCATCGATGAATT-CGAGCTCG-3′). Transformation of the resulting PCR product into W303 cells and selection for kanamycin resistance resulted in RJD3267, which contained a deletion of the entire coding sequence (as well as a partial deletion of the Ste6 gene, causing sterility in mating type a cells). The *UBA1* gene was amplified from wild-type W303 *Saccharomyces cerevisiae* cells by PCR using oligos NG19 (5′-CCGCTCGAGGGTAAAGTGGTTGAGCGAGGTA-3′) and NG20 (5′-GGACTAGTGAATTGCCATTACGCTTCC-3′; XhoI and SpeI sites underlined). The resulting PCR product, which contained *UBA1* coding sequences plus 186 base pairs upstream of the start codon and 269 base pairs downstream of the stop codon, was cloned into the CEN plasmid pRS316 to generate plasmid pRS316-UBA1. The diploid yeast strain RJD3267 was transformed with the pRS316-UBA1 plasmid, sporulation was induced, and a haploid strain was selected that contained both the deletion of *UBA1* and pRS316-UBA1 marked by *URA3*. A plasmid shuffling strategy was used to replace pRS316-UBA1 with pRS313-UBA1. The resulting strain, RJD3268, was used as the wild-type control strain in this study.

To generate mutant alleles of *UBA1*, random mutations were introduced into the *UBA1* gene by error-prone PCR by using the GeneMorph random mutagenesis kit (Stratagene, La Jolla, CA), NG19 and NG20 primers, and pRS316-UBA1 as the template. A gapped plasmid was created by digesting pRS313 with NheI. A uba1Δ haploid strain sustained by pRS316-UBA1 was cotransformed with the mutagenized PCR product and the gapped pRS313 plasmid and transformants were plated onto −His media. Cells that had lost the *URA* plasmid carrying wild-type *UBA1* were selected by replica plating onto plates containing 5-fluoro-orotic acid incubated at 25°C. Fifty temperature-sensitive mutants were identified by screening for colonies that grew at 25°C but not at 37°C.

Viability and Stress Sensitivity Assay

Wild-type and *uba1-204* yeast cells were grown to an optical density of A_{600} = 1. Ten-fold serial dilutions were spotted onto YPD plates and SD plates containing 30 μM $CdCl_2$. All plates were incubated at the indicated temperatures for 2–3 d.

Flow Cytometric Analysis of DNA Content

Cells were grown to log phase in YPD, washed, then incubated with 50 ng/ml α-factor for 2 h at 25°C to arrest cells in G1 phase. For the experiment in Figure 1C, an additional 25 ng/ml α-factor was added, and cultures were incubated at 37°C for 1 h. Cells were washed with YPD to reverse the α-factor arrest and resuspended in fresh YPD medium at 37°C. For the experiment in Figure 1D, an additional 25 ng/ml α-factor was added, and cells were kept at 25°C for 1 h. Cells were washed and grown in YPD at 25°C for 90 min to allow progression to G2 phase before shifting the cultures to 37°C.

Samples were withdrawn at indicated times after the shift to nonpermissive temperature, centrifuged at 13K rpm, and resuspended in 70% ethanol for 1 h at 4°C. The cell suspension was centrifuged for 5 min and washed with 50 mM sodium citrate. Cells were resuspended in 50 mM sodium citrate containing 0.25 mg/ml RNAse A and incubated at 50°C for 1 h. Cells were centrifuged and washed with phosphate-buffered saline, pH 7.2. Proteinase K was added to 2 mg/ml, and cells were incubated at 50°C for an additional hour. Propidium iodide was added at a final concentration of 16 μg/ml. Immediately before flow cytometric analysis, cells were sonicated twice at setting 3 for 3 s, and then they were filtered with Tekto mesh screen (Tekto, Elmsford, NY) into Falcon 2058 polystyrene tubes (BD Biosciences Discovery Labware, Bedford, MA).

Extract Preparation

Cells were harvested by centrifugation and drop frozen in liquid nitrogen. Cells were then resuspended in buffer containing 50 mM Tris, pH 7.5, 10% glycerol, 0.1% β-mercaptoethanol, and 1% SDS. An equal volume of glass beads (425–600 μm, acid washed; Sigma-Aldrich, St. Louis, MO) was added, and cell pellets were boiled for 3 min, vortexed in a ThermoSavant FastPrep (ThermoSavant, Holbrook, NY) for 45 s at a speed of 5.5, and boiled for an additional 2 min. Samples were resolved by SDS-polyacrylamide gel electrophoresis (PAGE), transferred to nitrocellulose, and probed with the indicated antibody. Antibodies were kindly provided by K. Madura (Robert Wood Johnson Medical School-UMDNY, Piscataway, NJ) for Rad23, M. Funakoshi (Kyushu University, Fukuoka, Japan) for Dsk2, R. Vierstra (University of Wisconsin-Madison, Madison, WI) for Rpn10, and J. Monaco (University of Cincinnati College of Medicine, Cincinnati, OH) for LMP. Ubiquitin was

detected with monoclonal antibody from Chemicon International (Temecula, CA).

Deg1-GFP Degradation

Cells harboring the Deg1-GFP plasmid (gift from R. Hampton, University of California, San Diego, La Jolla, CA) were grown in YP dextrose medium. Cultures of exponentially growing haploid yeast cells were incubated at 25°C or shifted to the nonpermissive temperature 37°C for 1 h, and cycloheximide was added to 0.5 mg/ml. Five-milliliter aliquots were harvested every 15 min, and extracts were prepared as above and analyzed with antiserum to green fluorescent protein (GFP) (Clontech, Mountain View, CA).

Ub^{V76}-V-βgal Degradation

Cells harboring the *GAL-Ub^{V76}-V-βgal* plasmid (gift from A. Varshavsky, California Institute of Technology, Pasadena, CA) were grown in SC raffinose medium −uracil. *Ub^{V76}-V-βgal* expression was induced at the permissive temperature for 1 h by the addition of galactose to 2%. Cultures were then incubated at either 25°C or shifted to 37°C for 1 h and transferred to fresh medium containing 2% dextrose to extinguish synthesis of Ub^{V76}-V-βgal. Portions were withdrawn every 15 min and processed for immunoblotting with antiserum to β-galactosidase.

Sic1 Degradation

Cells grown in SC raffinose medium −uracil were arrested with 50 ng/ml α-factor for 1 h, and *GAL-SIC1* expression was induced transiently for 1 h by the addition of galactose to 2%. Supplemental α-factor was added to 25 ng/ml when cells were shifted to 37°C for 1 h. Cells were then washed and transferred to fresh medium containing 2% dextrose to quench *SIC1* expression, and portions were withdrawn every 30 min and processed for immunoblotting with antiserum to Sic1 (gift from L. Johnston, National Institute for Medical Research, London, United Kingdom).

Native Gel Activity Assay

26S proteasome samples were purified as described below and resolved by nondenaturing PAGE as described in Glickman *et al.*, 1998. The gel was incubated with fluorogenic peptide Suc-LLVY-AMC for 10 min at 30°C. Proteolytic activity of the resolved complexes was visualized by exposure to UV light. Protein complexes in the same gel were also detected with Coomassie blue stain.

In Vitro Ub-Sic1 Degradation

Ub-Sic1 was synthesized as described in Saeki *et al.*, 2005. Briefly, 0.5 μM Ub-Sic1 was incubated with 1 μM 26S proteasome in the presence of 3 mM magnesium acetate and 1X ATP regenerating system (ARS) (Verma *et al.*, 1997) for 0 or 5 min as specified. Proteasomes treated with 100 μM epoxomicin were preincubated with drug at 30°C for 30 min before addition of Ub-Sic1. Reactions were terminated by adding SDS Laemmli buffer, and samples were resolved by SDS-PAGE, blotted to nitrocellulose, and visualized with antibody to Sic1 (gift from L. Johnston).

Preparation of Extracts for Affinity Purification of 26S Proteasomes

Yeast strains were grown to log phase at 25°C in medium containing 0.67% yeast nitrogen base minus amino acids, 2% dextrose, 0.5% casamino acids, and 20 mg/l adenine and tryptophan. Cultures were split in half, and 25°C cultures were incubated for 40 min at 25°C, whereas 37°C cultures were pelleted by centrifugation, resuspended in fresh 37°C medium, and incubated at 37°C for 40 min. Cells were pelleted by centrifugation for 5 min at 4°C for 25°C samples and at 37°C for 37°C samples. Pellets were washed with 25 or 37°C sterile water and frozen in liquid nitrogen for a minimum of 2 h. Frozen cell pellets were ground to a fine powder under liquid nitrogen in a mortar placed on a bed of dry ice. Powder was resuspended in 1 pellet-volume of column buffer containing 50 mM Tris, pH 7.5, 150 mM NaCl, 10% glycerol, 5 mM $MgCl_2$, and 5 mM ATP. ARS (Verma *et al.*, 1997) was added, and the lysates were clarified by centrifugation at 17,000 rpm for 20 min.

Lysates were supplemented again with 5 mM ATP and 5 mM $MgCl_2$ and incubated at 4°C for 2 h with FLAG antibody-coupled beads from Sigma-Aldrich that had been prewashed in 0.1 M glycine, pH 3.5, and resuspended as a 50% slurry with column buffer. For each sample, 1.5 ml of lysate was incubated with 1 ml of bead slurry. Bead-bound proteins were pelleted and washed three times with high-salt wash buffer consisting of the column buffer described above supplemented with 0.2% Triton X-100 and NaCl to a final concentration of 200 mM.

For Coomassie blue staining, immunoprecipitations, and native gel experiments, bead-bound proteins were then washed twice with low-salt wash buffer containing 50 mM Tris-HCl, pH 7.5, 20 mM $MgCl_2$, and 2 mM ATP. All supernatant was aspirated with a 25-gauge needle, and the pellet was resuspended in 3 times its volume of elution buffer containing 25 mM Tris, pH 7.5, 150 mM NaCl, 5 mM $MgCl_2$, 2 mM ATP, and 100 μg/ml Flag peptide, and proteins were eluted at 4°C for 3 h. Samples were analyzed by SDS-PAGE on a 4–12% Tris-glycine gel (Invitrogen, Carlsbad, CA).

For proteasome-binding experiments in Figure 6, A–C, bead-bound proteasome complexes were resuspended in column buffer and incubated with 1 mM phenanthroline, 2.5 μM ubiquitin-aldehyde (Boston Biochem, Cambridge, MA), 100 μM MG132, 1 mM ATP, and 5 mM $MgCl_2$ on ice for 1 h. Purified proteins were added as indicated, and samples were incubated on ice for 1 h. GST-Rad23, GST-UbL, GST-UBA, and Rad23-HIS were gifts from R. Verma (Caltech, Pasadena, CA), Sic1 was a gift from G. Kleiger (Caltech), and tetra-ubiquitin (Ub_4) was from Boston Biochem. The bound fraction was washed three times with high-salt wash buffer, twice with low-salt wash buffer, supernatant was aspirated, and proteins were eluted with SDS loading buffer and analyzed by SDS-PAGE on a gradient gel.

MG132-binding Assay

Yeast strains that were sensitive to proteasome inhibitors were generated by deletion of the *pdr5* gene. RJD3269 (wild type; WT) and RJD3270 (*uba1-204*) were grown in casamino acids medium at 25°C and MG132 (American Peptide, Sunnyvale, CA) or dimethyl sulfoxide (DMSO) was added. After 5 min, 37°C medium was added to shift cells to the nonpermissive temperature, and cultures were incubated at 37°C for 1 h. Cells were centrifuged at 37°C for 5 min and flash frozen. 26S proteasome complexes were purified as described above.

RESULTS

Isolation and Phenotypic Analysis of uba1-204

The initial goal of this study was to create a loss-of-function allele of the *S. cerevisiae* ubiquitin-activating enzyme that resulted in strong and rapid inactivation of ubiquitin conjugation. To generate mutant alleles, the *UBA1* coding sequence, carried on a *HIS3* plasmid and expressed from the natural *UBA1* promoter, was subjected to random PCR mutagenesis. Mutant plasmids were introduced into *uba1*Δ haploid cells sustained by a low copy *URA3* plasmid containing *UBA1*. Transformants were plated on 5-fluoro-orotic acid to evict the *UBA1* plasmid, and clones sustained by the mutagenized plasmid were sought. In a screen for temperature-sensitive growth, 50 candidate mutant alleles that grew at 25°C but failed to grow at 37°C were isolated. To identify mutants that exhibited broad defects in the ubiquitin–proteasome system, we tested these alleles for loss of ubiquitin conjugates at the nonpermissive temperature. Whereas the majority of these *uba1* mutants largely retained ubiquitin conjugates at the nonpermissive temperature, *uba1-204* mutant cells rapidly lost ubiquitin conjugates upon transfer to 37°C (data not shown). The *uba1-204* mutant gene was sequenced and found to contain mutations leading to eight amino acid alterations: D318G, K401E, V502A, E523G, E534G, N630G, F680S, and D936E. Although none of the mutations occurred in proximity to the adenylation site or the active site cysteine, four mutations resulted in the replacement of acidic residues with glycine.

Cellular viability was unaffected in *uba1-204* cells grown at temperatures ranging from the 25°C permissive temperature up to 33°C (Figure 1A). Mutant cells began to exhibit loss of viability at 35°C, and they were completely inviable at 37°C (Figure 1A). Sensitivity to environmental stress is often a hallmark of ubiquitin pathway mutations because stress leads to an increased requirement for protein quality control. As predicted, compromised Uba1 activity conferred sensitivity to the cellular stressor cadmium chloride (Figure 1B). Another important feature of the UPS is its critical role in the regulation of cell cycle progression. Whereas mammalian E1 mutants arrest at S/G2 (Finley *et al.*, 1984; Kulka *et al.*, 1988), the cold-sensitive yeast *uba1-165* mutant has a G1 arrest phenotype (Cheng *et al.*, 2002) and existing temperature-sensitive yeast E1 mutants do not display a cell cycle phenotype (McGrath, 1991; Swanson and Hochstrasser, 2000). In *uba1-204* mutants, cellular division ceased within one cell cycle of shifting cells to the nonpermissive

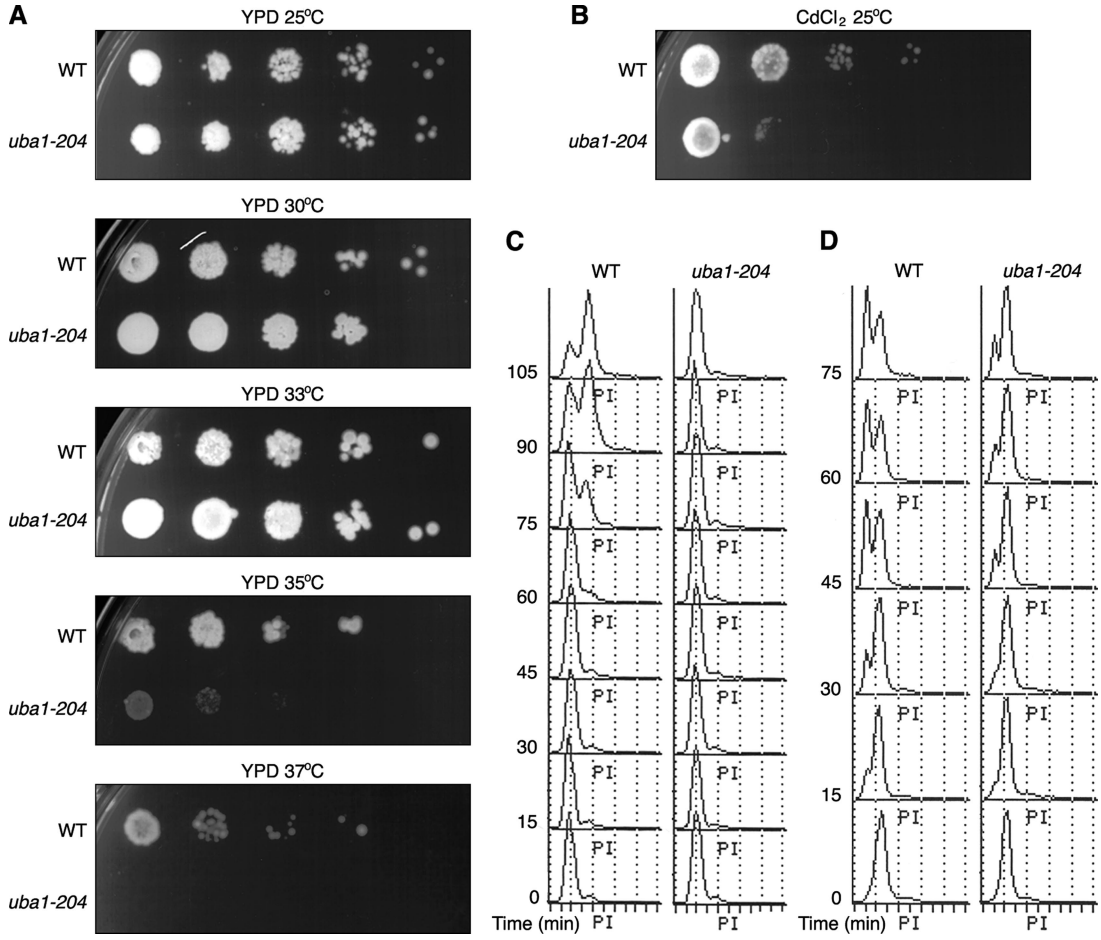

Figure 1. *Uba1-204* cells are temperature-sensitive and undergo cell cycle arrest. Ten-fold serial dilutions of wild-type and *uba1-204* yeast cells were spotted onto YPD (A) and SD plates containing 30 μM $CdCl_2$ (B) and incubated at the indicated temperatures for 2–3 d. (C) Wild-type and *uba1-204* cells were arrested with α-factor for 2 h at the permissive temperature. The temperature was shifted to 37°C for 1 h, and the cells were released into fresh medium at 37°C at time 0. At indicated time points after release, aliquots were withdrawn and prepared for assessment of total DNA content by fluorescence-activated cell sorting. (D) WT and *uba1-204* cells were arrested with α-factor for 3 h at the permissive temperature. Cells were then released into fresh medium and grown at 25°C for 90 min to allow progression to G2 phase. Cells were then shifted to 37°C at time 0, and aliquots were withdrawn and analyzed as described in C.

temperature (data not shown). To test the influence of the *uba1-204* mutation on the cell cycle, cellular DNA content was assessed by flow cytometric analysis. When unsynchronized *uba1-204* cultures were incubated at the nonpermissive temperature, a G2 arrest phenotype was apparent, with some cells continuing through the cell cycle to arrest in G1 (data not shown). Next, we synchronized cells in G1 with α-factor before shifting them to 37°C. On release into fresh medium at the nonpermissive temperature, wild-type cells progressed through the cell cycle, whereas *uba1-204* cells remained arrested at G1 (Figure 1C). By contrast, when α-factor-synchronized cells were released into fresh medium at the permissive temperature for 90 min to allow progression through the cell cycle before shifting to 37°C, *uba1-204* cells arrested at G2, whereas wild-type cells did not (Figure 1D). Therefore, *uba1-204* mutant cells arrest at both G1 and G2 of the cell cycle.

Uba1-204 Cells Are Defective in Ubiquitin Conjugation and Substrate Degradation

Complete inactivation of Uba1 should yield universal shutdown of downstream ubiquitination pathways. To determine the extent of impairment to the UPS, the effect of the ubiquitin activation defect on overall ubiquitin conjugate accumulation was monitored. Strikingly, a nearly complete loss of detectable ubiquitin conjugates was observed within 5 min of shifting the cells to 37°C (Figure 2A). The temperature-dependent defect in ubiquitin conjugate accumulation was partially reversed when cell cultures that were incubated at 37°C for 1 h were returned to 25°C (Figure 2B). To determine the impact of this reversibility on future experiments, we evaluated the restoration of ubiquitination activity in mutant cells incubated on ice. Unexpectedly, it was observed that in *uba1-204* cultures that were incubated at 37°C and then placed on ice, ubiquitin conjugates began to reappear within minutes (Figure 2C). Therefore, to ensure full Uba1 inactivation in all future experiments, wild-type and mutant cells were maintained at the restrictive temperature throughout centrifugation and preparation steps until they were flash-frozen in liquid nitrogen.

The dramatic reduction in ubiquitin conjugate levels in *uba1-204* cells at the nonpermissive temperature indicates that multiple downstream E2/E3 pathways were inactivated. If this was indeed the case, stabilization of diverse proteasome

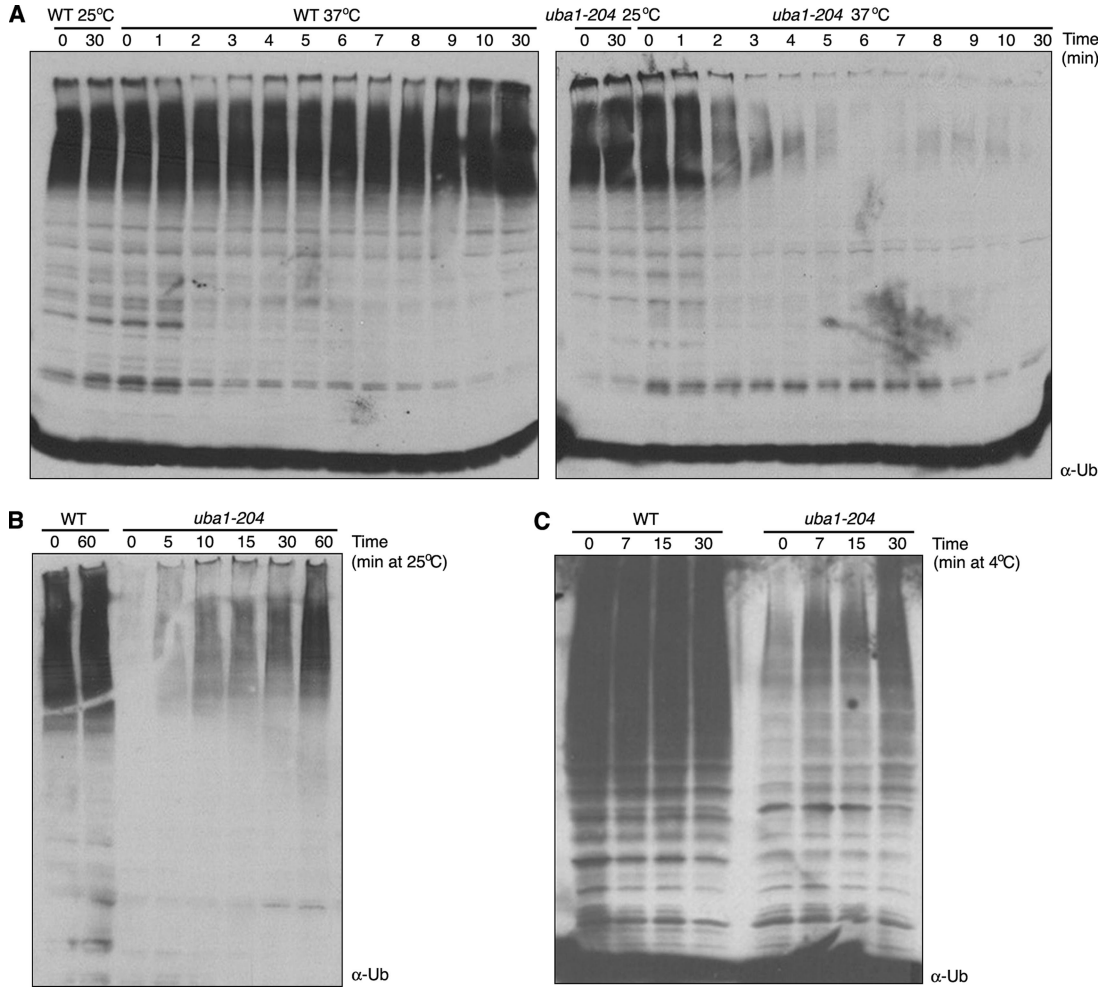

Figure 2. *Uba1-204* cells are defective in ubiquitin conjugation. (A) Wild-type and *uba1-204* cells were grown to log phase in liquid YPD medium at 25°C. Half of each culture was shifted to the nonpermissive temperature at time 0, and samples were withdrawn every minute. Lysates were prepared and analyzed by SDS-PAGE and immunoblotted with antiserum to ubiquitin. (B) Loss of ubiquitin conjugation is reversible. Strains were grown as described in A, and samples were shifted to the nonpermissive temperature for 1 h. At time 0, the temperature was shifted back to 25°C, aliquots were withdrawn at indicated time points, and ubiquitin conjugation was assessed as described in A. (C) *Uba1-204* cells are active in ubiquitin conjugation at 4°C. Cells were grown in YPD medium and shifted to 37°C for 1 h. At time 0, ice was added to cultures, and the temperature was shifted to 4°C. Samples were withdrawn and analyzed as described in A.

substrates would be expected. To test this, the turnover of a synthetic substrate, Deg1-GFP, was analyzed in a cycloheximide chase experiment. Deg1-GFP bears the degradation signal from the rapidly degraded Matα2 protein and is a substrate for the ubiquitin ligase Doa10 (Swanson *et al*., 2001). At 25°C, the majority of Deg1-GFP was destroyed within 15 minutes of terminating protein synthesis in both wild-type and mutant strains (Figure 3A). The protein was modestly stabilized in the wild-type cells at 37°C, but it was completely stabilized in the *uba1-204* cells at 37°C. To examine the scope of this proteolytic defect, we also monitored turnover of Ub^{V76}-V-βgal, a cytosolic substrate of the UFD ubiquitin–ligase pathway (Johnson *et al*., 1995). Although degradation of Ub^{V76}-V-βgal occurred with normal kinetics following a galactose promoter shutoff in the mutant cells at 25°C, there was no detectable degradation of the substrate in *uba1-204* cells at 37°C (Figure 3B). In addition, ubiquitin–protein conjugates could be detected in the early time points of the wild-type samples at both temperatures and in the mutant samples at 25°C. However, these conjugates were conspicuously absent in *uba1-204* cells at 37°C.

To determine the physiological significance of these turnover defects, we also assessed the effect of impaired ubiquitin activation on a well-characterized endogenous substrate, the cyclin-dependent kinase inhibitor Sic1. At the G1/S boundary, Sic1 is polyubiquitinated by the SCF ubiquitin ligase and degraded by the proteasome (for review, see Deshaies, 1999). Wild-type and mutant cells carrying a *GAL-SIC1* allele were arrested with α-factor, Sic1 expression was transiently induced by a pulse of galactose, and cells were released from G1 into the cell cycle. Similarly to the test substrates, Sic1 degradation was not impaired in *uba1-204* cells at the permissive temperature, but the protein was stabilized at 37°C (Figure 3C). These data confirm that in the *uba1-204* mutant, multiple downstream proteolytic pathways were inhibited.

Uba1-204 Cells Contain Intact and Active Proteasomes

To address the impact of a ubiquitin activation defect further downstream in the ubiquitination pathway, we examined its affect on the proteasome. Considering that many subunits of

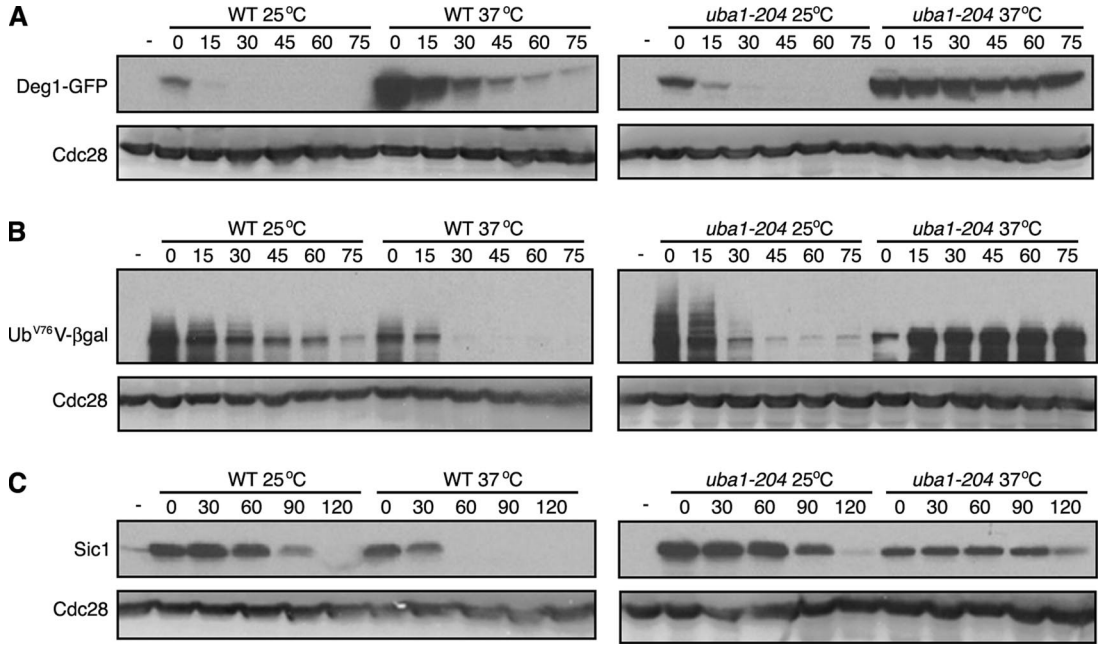

Figure 3. *Uba1-204* cells are defective in substrate degradation. (A) Wild-type and *uba1-204* cells expressing Deg1-GFP were grown in SD medium. Cultures of exponentially growing cells were incubated at 25°C or shifted to 37°C for 1 h, and cycloheximide was added to initiate a chase period. Samples were withdrawn every 15 min for analysis. Lysates were separated by SDS-PAGE and immunoblotted with antiserum to GFP. Control (−) sample was an isogenic strain lacking the plasmid. (B) WT and *uba1-204* cells expressing Ub^{V76}-V-βgal under control of the *GAL1* promoter were grown in SD medium with raffinose, and protein expression was induced by addition of 2% galactose for 1 h. Cultures were then incubated at either 25°C or shifted to 37°C for 1 h and transferred to dextrose medium pre-equilibrated at the same temperature to initiate a chase. Samples were withdrawn every 15 min, lysed, separated by SDS-PAGE, and immunoblotted with antiserum to β-gal. (C) WT and *uba1-204* cells expressing Sic1 under the control of the *GAL1* promoter were grown in SD medium with raffinose, and they were arrested with α-factor for 2 h. Sic1 expression was induced by addition of galactose to 2%, and cells were incubated at 25°C for 1 h before shifting to 37°C for an additional hour. Cells were then transferred to dextrose medium to initiate a chase, and samples were withdrawn every 30 min, lysed, separated by SDS-PAGE, and immunoblotted with antiserum to Sic1. Protein loading was verified by immunoblotting with antiserum to Cdc28.

the proteasome can be ubiquitinated (Peng *et al*., 2003) and the proteasome has been suggested to undergo rapid cycles of assembly/disassembly (Babbitt *et al*., 2005), we first investigated a possible role for polyubiquitination in the maintenance of fully-assembled 26S proteasome complexes. When the chromosomal locus encoding Pre1, an alpha subunit of the 20S core, is replaced by an allele tagged with a Flag epitope, it is possible to obtain intact, active 26S proteasome complexes by a single-step affinity purification method (Verma *et al*., 2000). Proteasomes affinity-purified from *uba1-204* cells cultured at either 25 or 37°C were found to contain all 20S and 19S subunits with no detectible change in subunit composition (Figure 4A). Quantitative mass spectrometric analysis of these samples also showed no significant alteration in subunit abundance in proteasomes purified from the mutant strain (data not shown). The assembly state of the purified proteasomes was further confirmed by subjecting samples to native gel electrophoresis. Coomassie blue staining showed intact 20S core particle (CP) complexes as well as proteasomes with either one (R_1P) or two (R_2P) 19S regulatory particles attached (Figure 4B). The ratios of various proteasomal subcomplexes in the mutant were comparable with that seen in wild type. Thus, a robust level of ongoing ubiquitination is not necessary to sustain proteasome complexes in vivo, making it possible to purify intact proteasomes from *uba1-204* mutant cells.

Next, we examined the functional consequences of E1 inactivation on these intact proteasomes. Proteasome peptidase activity was tested by in-gel hydrolysis of the fluorogenic peptide substrate Suc-LLVY. Peptidase activity was similar for 20S and 26S proteasomes purified from wild-type and *uba1-204* cells (Figure 4C). Purified 26S proteasomes were next evaluated for their ability to degrade a polyubiquitinated physiological substrate, Sic1 (Ub-Sic1). High-molecular-weight Ub–Sic1 conjugates were completely degraded upon incubation with proteasomes isolated from wild-type or *uba1-204* cells (Figure 4D). Inhibition of proteolysis with the 20S proteasome inhibitor epoxomicin resulted in accumulation of deubiquitinated Sic1 (Figure 4D) (Verma *et al*., 2002).

Ubiquitin-binding Proteins Have Differential Requirements for Polyubiquitin in Proteasome Targeting

Having established that cellular depletion of polyubiquitin chains does not alter proteasome activity, we next evaluated the association of known ubiquitin-binding factors with the proteasome. Cell lysates were prepared from wild-type and mutant cells, and proteasome complexes were purified by immunoprecipitation as described above. Comparable levels of the 20S proteasome subunit Pre2 (tested with antibody to LMP7, the human homolog) were detected in all immunoprecipitation samples, verifying that equivalent amounts of intact proteasomes were purified from wild-type and *uba1-204* cells (Figure 5E). As expected, there was a striking decrease in the association of ubiquitin conjugates with the 26S complex isolated from *uba1-204* cells held at 37°C (Figure 5A). Although Rpn10 is the only validated ubiquitin-binding proteasome subunit, most cellular Rpn10 remains free, unincorporated into proteasome complexes (van Nocker and Vierstra, 1993; Deveraux *et al*., 1994; Haracska and Udvardy, 1995). This had suggested the

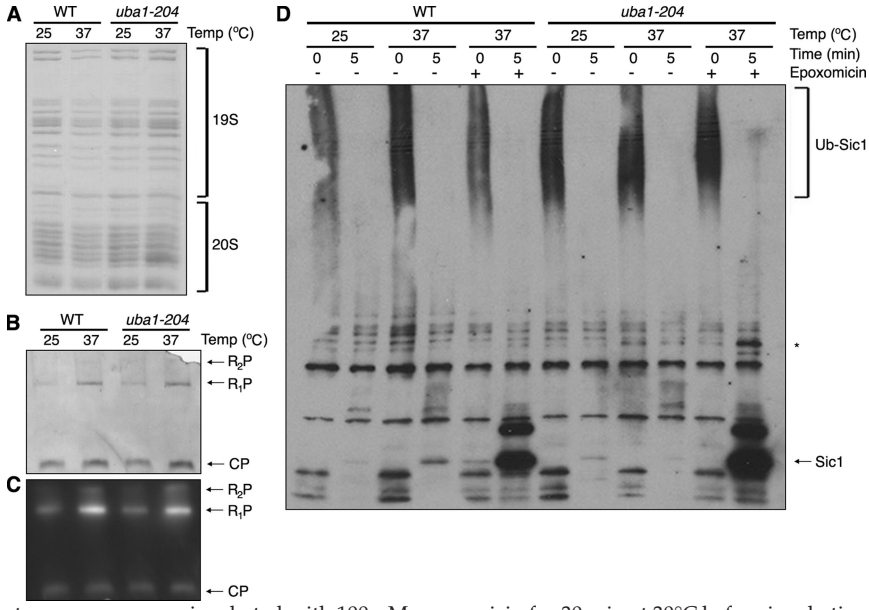

Figure 4. 26S proteasomes isolated from *uba1-204* cells are properly assembled and proteolytically active. (A) Intact 26S proteasomes can be purified from *uba1-204* cells. Wild-type and *uba1-204* cells were incubated at 25 or 37°C for 40 min, and 26S proteasomes were affinity purified as described in *Materials and Methods*. Samples were resolved by SDS-PAGE and visualized by Coomassie blue staining. 19S and 20S subunits are specified. (B) Purified proteasome complexes were resolved by nondenaturing PAGE and were visualized by Coomassie blue staining. CP refers to 20S core particle, and R_1P and R_2P refer to 26S proteasomes with one or two regulatory caps, respectively. (C) Proteolytic activity of the complexes in the nondenaturing gel from B was detected by fluorogenic peptide overlay with the peptidase substrate Suc-LLVY-AMC. Fluorescent bands were visualized by exposure to UV light. (D) *Uba1-204* 26S proteasomes are active in the degradation and deubiquitination of Ub-Sic1 in vitro. 26S proteasomes purified from wild-type and *uba1-204* cells were incubated with Ub-Sic1 at 30°C for 5 min. For deubiquitination reactions, proteasomes were preincubated with 100 μM epoxomicin for 30 min at 30°C before incubation with Ub-Sic1. Samples were analyzed by SDS-PAGE followed by immunoblotting with anti-Sic1 antibody. Polyubiquitinated Sic1 (Ub-Sic1) and deubiquitinated Sic1 are specified. There is a band of unknown identity (*) that was reproducibly detected in the mutant but not wild-type lanes in the presence of epoxomicin.

possibility that Rpn10 may form transient interactions with the proteasome, cycling between a free and assembled form. We therefore examined how the defect in ubiquitination impacted this potential equilibrium. Analysis of purified proteasomes indicated that loss of polyubiquitination activity in *uba1-204* cells did not alter Rpn10 incorporation into the proteasome complex (Figure 5D). These data, verified by mass spectrometry, suggest that the interaction of Rpn10 with polyubiquitin chains is not necessary for its association with intact proteasome complexes. In contrast, proteasomes isolated from *uba1-204* at the restrictive temperature exhibited a significant decrease in the amount of associated UbL/UBA proteins Rad23 (Figure 5B) and Dsk2 (Figure 5C). Meanwhile, overall levels of both Rad23 and Dsk2 proteins in mutant cell lysates were not affected by the temperature shift. Therefore, whereas both UbL/UBA receptor proteins were indeed present in cell lysates, they failed to associate efficiently with the proteasome upon inhibition of ubiquitin conjugate formation.

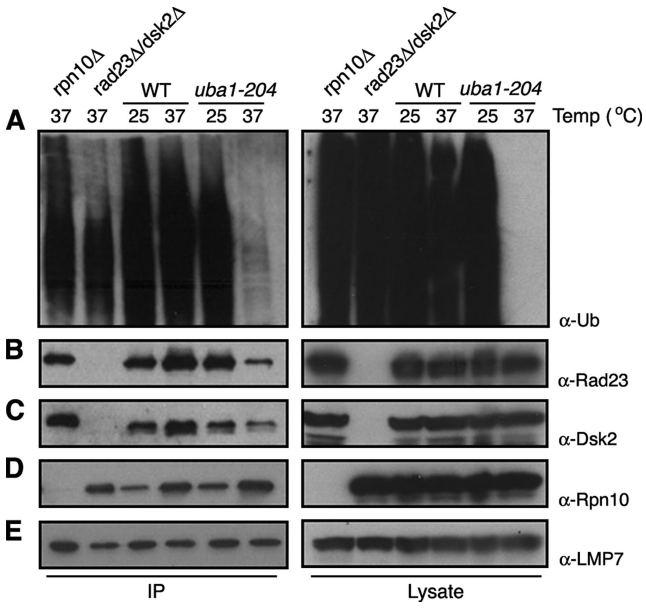

Figure 5. 26S proteasomes isolated from *uba1-204* cells exhibit reduced content of UbL/UBA proteins. (A–E) Wild-type and *uba1-204* cells expressing Pre1-FH were grown in casamino acid medium, and half the cultures were shifted to 37°C for 40 min. Lysates were prepared and immunoprecipitated as described in *Materials and Methods* by incubation with anti-Flag resin in the presence of ATP. Intact 26S proteasomes were eluted with Flag peptide, separated by SDS-PAGE, and proteasome-bound proteins were detected by immunoblotting with antiserum to the specified proteins. Antibody specificity was verified using deletion mutants.

Ubiquitin Chains Promote Rad23 Association with the Proteasome

To examine the nature of ubiquitin conjugation-dependent receptor targeting in more depth, we focused on Rad23. The dual recognition capability of UbL/UBA proteins such as Rad23 underlies their adaptor function, but it is unclear whether ubiquitin chain binding occurs before or after proteasome binding. The essentially ubiquitin-free nature of proteasomes purified from *uba1-204* cells presented an ideal reagent with which to examine the ubiquitin dependence of Rad23 targeting. If the observed decrease in Rad23 interaction with the proteasome was truly a result of its inability to locate ubiquitinated substrates, addition of such substrates would be expected to restore binding. To test this hypothesis, we analyzed the effect of addition of the polyubiquitinated substrate Ub-Sic1 on the ability of GST-Rad23 to bind to immobilized proteasomes in vitro. As the results in Figure 6A illustrate, addition of Ub-Sic1 resulted in a marked increase in the binding of full-length GST-Rad23 to proteasomes. A GST–UBA fusion protein that lacks the proteasome-binding UbL domain was unable to associate with the proteasome. Conversely, Rad23 lacking the UBA domains (GST-UbL) binds the proteasome regardless of whether Ub–

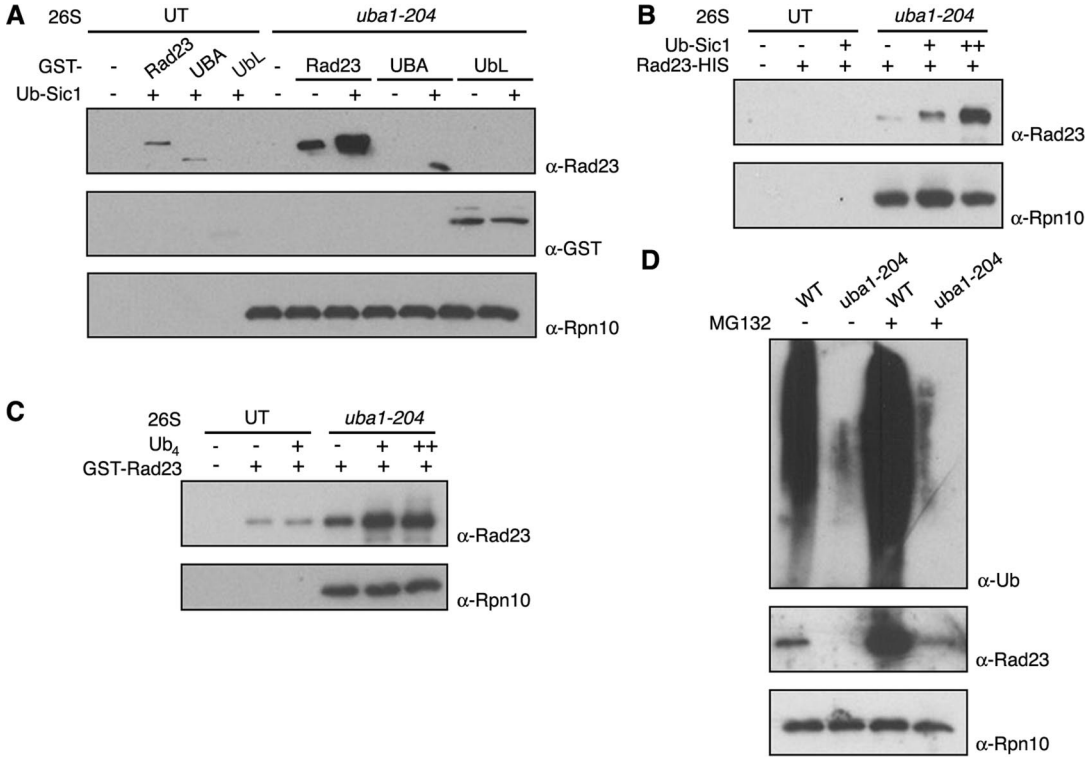

Figure 6. Polyubiquitin chains promote Rad23 binding to the proteasome. (A) Ub-Sic1 promotes association of full-length GST-Rad23 with proteasome. Sepharose-immobilized proteasomes isolated from *uba1-204* cells were preincubated with proteasome inhibitors for 1 h and then incubated with GST fusion proteins in the presence or absence of Ub-Sic1 as described in *Materials and Methods*. GST-Rad23 refers to the full-length protein, whereas GST-UBA contains both UBA domains and lacks the UbL domain. GST-UbL contains only the N-terminal UbL domain and was detected with antiserum to GST. The presence of proteasome complexes was verified with antibodies to Rpn10. As a negative control, simultaneous purification was conducted with *uba1-204* cells lacking the Pre1-Flag allele (UT). (B) Ub-Sic1 promotes association of Rad23-HIS with proteasome. Immobilized proteasomes were prepared as described in A and incubated with Rad23-HIS in the presence or absence of Ub-Sic1. (C) Free tetraubiquitin chains are sufficient to promote GST–Rad23 association. Immobilized proteasomes were prepared as described in A and incubated with GST-Rad23 and Ub_4 rather than Ub-Sic1. (D) Stabilization of polyubiquitin conjugates in vivo promotes Rad23 targeting to the proteasome. Wild-type and *uba1-204* cells were treated with MG132 or DMSO (−) and shifted to 37°C for 1 h. Proteasomes were isolated by affinity purification, ubiquitin conjugates were detected with antiserum to ubiquitin, and Rad23 binding was assessed.

Sic1 substrate is present (Figure 6A). The Ub-Sic1 dependence of GST-Rad23 targeting provides further support for the notion that Rad23 interaction with polyubiquitinated proteins can promote its shuttling to the proteasome. Furthermore, to ensure that this response was independent of the glutathione *S*-transferase (GST) tag, Rad23 fused to a His_6 epitope was also tested with similar results (Figure 6B).

A recent report demonstrated that Ub-Sic1 is preferentially delivered to the proteasome by either Rad23 or Rpn10 (Verma *et al*., 2004). The capacity of Rad23 and other receptors to selectively target substrates for proteolysis suggests that in addition to recognizing conjugated polyubiquitin chains, Rad23 may detect degradation signals within substrate proteins themselves. To determine whether recognition of a polyubiquitin chain is sufficient to enhance binding of Rad23 to the proteasome, free K48-linked Ub_4 chains were tested in the proteasome-binding assay. The results in Figure 6C demonstrate that Ub_4 was sufficient to stimulate GST-Rad23 recruitment to the proteasome and that the presence of conjugated substrate is not absolutely required to drive targeting in vitro. To extend these conclusions to a cellular environment, the proteasome inhibitor MG132 was used to stabilize polyubiquitin chains in vivo. Analysis of proteasomes from cells treated with the drug confirmed an increase in proteasome-bound Rad23 in both mutant and wild-type cells coincident with an increase in ubiquitin chains (Figure 6D).

DISCUSSION

The initial goal of this study was to develop a method of inhibiting the entire ubiquitin–proteasome system in the budding yeast *S. cerevisiae*. A single budding yeast E1, Uba1, initiates an intricate downstream ubiquitination network consisting of 11 E2s and 54 potential E3s that act on a multitude of proteins, marking them for destruction. At the apex of the entire pathway, the essential E1 gene *UBA1* is the logical target to disrupt all downstream ubiquitination reactions. Indeed, experiments conducted with a mouse cell line harboring a temperature-sensitive E1 provided the foundation for fundamental early discoveries on the nature of the UPS (Ciechanover *et al*., 1984; Finley *et al*., 1984; for review, see Pickart and Cohen, 2004). Yet, although conditional mutants of the *S. cerevisiae* E1 gene had previously been isolated, they only exhibit partial loss-of-function phenotypes, limiting their utility (Swanson and Hochstrasser, 2000; McGrath *et al*., 1991; Cheng *et al*., 2002). We reasoned that a strong, fast-acting, temperature-sensitive *uba1* mutant would be a valuable tool to address the role of ubiquitin conjugation in myriad processes. We show that it is possible to isolate such

a mutant and, as a demonstration of its utility, we have used the *uba1-204* mutant to explore the nature of ubiquitin chain recognition and delivery pathways.

Phenotypic characterization of the *uba1-204* mutant revealed that it exhibited dramatic loss of both polyubiquitin conjugates and proteolysis at the restrictive temperature. On shifting mutant cells to 37°C, nearly all detectable ubiquitin conjugates disappeared in a matter of minutes, suggesting rapid inactivation of the mutant Uba1 protein. Moreover, the turnover of three proteins targeted by different ubiquitin ligases was severely inhibited in mutant cells held at the nonpermissive temperature. Together, the rapid loss of conjugates and the stabilization of three different UPS substrates indicate the interruption of multiple downstream proteolytic pathways. However, it is possible that there remains a level of ubiquitin activation sufficient to sustain some degradation pathways. Clb2 did not accumulate in G1-arrested *uba1-204* cells upon induction of its expression from a *GAL* promoter, whereas Clb2 lacking its destruction box did accumulate (A. Amon, personal communication). This observation suggests that anaphase-promoting complex-dependent degradation of Clb2 may continue despite the otherwise severe defects seen in *uba1-204*. It will be interesting in the future to determine the proteome-wide effects of this mutation.

To investigate the impact of polyubiquitin chain depletion on substrate delivery processes, we examined the ubiquitin–proteasome system at its terminus, the proteasome. We present evidence that although robust ubiquitination is not required for the maintenance of functional 26S proteasomes, it may regulate the recruitment of some ubiquitin-binding proteins. In the absence of normal ubiquitination, Rpn10 incorporation into the proteasome complex was unaltered. However, in extracts from *uba1-204* mutant cells, proteasomes were associated with reduced levels of the UbL/UBA proteins Rad23 and Dsk2.

Two models have been proposed to describe the mechanism of Rad23-mediated substrate delivery (for review, see Madura, 2004). According to the "shuttle factor" model, Rad23 first recognizes polyubiquitinated proteins via its UBA domains and subsequently shuttles substrates to the 26S proteasome. In contrast, the "alternative receptor" model predicts that Rad23 docks onto the proteasome via its UbL domain and acts alongside Rpn10 as a receptor for trapping polyubiquitinated substrates. Because they can be isolated largely free of associated ubiquitin chains, proteasomes from *uba1-204* cells provide a means of distinguishing between these two models by addressing the key question of whether Rad23 association with the proteasome depends on the availability of ubiquitinated substrates. A decrease in proteasome-bound Rad23 was observed in the *uba1-204* mutant. Moreover, the results in Figure 6 demonstrate that addition of polyubiquitin chains (either unanchored or substrate-bound) to a polyubiquitin-free in vitro system promoted Rad23 association with the proteasome. The defect in Rad23 targeting could also be rescued in vivo by increasing cellular polyubiquitin levels with the proteasome inhibitor MG132. Based on these results, we propose that the recognition of ubiquitin chains typically precedes (and activates) proteasomal targeting of Rad23. Conversely, if detection methods accurately reflect a near absence of ubiquitin conjugates in the mutant, then the remaining Rad23 residing on proteasomes isolated from these cells hints that Rad23 can operate as postulated by the alternative receptor model, albeit less efficiently than it functions as a shuttling factor.

Recent findings have suggested that intramolecular interactions can regulate Rad23 activity, resulting in a "closed conformation" and steric inhibition of the receptor. In hHR23a, the human homologue of Rad23, the UbL domain engages in a transient intramolecular interaction with both UBA domains (Walters *et al*., 2003). NMR spectroscopic analysis of yeast Rad23 has revealed that although the first UBA domain can participate in an intramolecular interaction with the UbL, the carboxy-terminal UBA domain does not (Kang *et al*., 2006). Intramolecular interactions could result in inhibition of intermolecular activity such as proteasome and ubiquitin chain binding. This may explain the finding that full-length Rad23 has a lower affinity for the proteasome than does its truncated UbL domain (Elsasser *et al*., 2002) and removal of the UbL domain from intact hHR23a increases its affinity for free Ub_6 chains four-fold (Raasi *et al*., 2004). These biophysical data predict that addition of ubiquitin conjugates should stimulate Rad23 association with ubiquitin-depleted proteasomes in vitro and that depletion of ubiquitin conjugates in vivo should lead to reduced association of Rad23 with the proteasome whereas accumulation of ubiquitin conjugates should enhance Rad23 association with the proteasome. All three of these predictions have been confirmed in this report. Together, the data suggest that in its "resting" state, Rad23 is in a closed conformation in which the UbL and first UBA domain engage in an intramolecular association. On coming into contact with substrate, the UBA domain preferentially binds the substrate's ubiquitin chain owing to its 10-fold higher affinity for ubiquitin compared with UbL (Ryu *et al*., 2003). The exposed UbL domain can now bind to the proteasome. Although binding of Rad23's UBA domains to polyubiquitin and its UbL domain to proteasome may be sequential, the process is not necessarily unidirectional. It is possible that disruption of the weak UbL–UBA intramolecular interaction could occur either through UBA recognition of a polyubiquitin chain or through binding of UbL to proteasome, which could account for the presence of low amounts of Rad23 on proteasomes purified from *uba1-204* cells. Nevertheless, whether this binding is truly due to docking of "empty" receptor molecules or is merely an artifact of residual Uba1 activity, our data clearly demonstrate that ubiquitin chains significantly enhance stable association of Rad23 with the proteasome in vitro and in vivo.

In summary, we have isolated and characterized a new allele of budding yeast ubiquitin-activating enzyme that results in nearly complete inhibition of the ubiquitin–proteasome pathway. Our data reveal that although ongoing ubiquitination is not necessary for proteasome activity, ubiquitin conjugates promote the association of ubiquitin-binding substrate receptor proteins with the proteasome. In addition to regulated proteolysis, ubiquitin is involved in a broad range of other cellular functions, including cell cycle control, transcription, DNA repair, signal transduction, and endocytosis. Mutant *uba1-204* cells will be a valuable tool in further exploration of ubiquitin-dependent proteolysis as well as the many other ubiquitin-dependent cellular processes.

ACKNOWLEDGMENTS

We thank members of the Deshaies laboratory, especially R. Verma and R. Oania, for reagents and helpful discussions and R. Verma for critical reading of the manuscript. We gratefully acknowledge R. Diamond and P. Koen (Caltech Cell Sorting Facility) for performing flow cytometry and J. Graumann, T. Mayor, and G. Smith (Caltech) for mass spectrometry analysis. We thank A. Varshavsky, R. Hampton, K. Madura, M. Funakoshi, R. Vierstra, L. Johnston, and J. Monaco for generously providing reagents. R.D. is an investigator of the Howard Hughes Medical Institute, and this work was supported by Howard Hughes Medical Institute.

REFERENCES

Amerik, A. Y., and Hochstrasser, M. (2004). Mechanism and function of deubiquitinating enzymes. Biochem. Biophys. Acta *1695*, 189–207.

Babbitt, S. E. *et al*. (2005). ATP hydrolysis-dependent disassembly of the 26S proteasome is part of the catalytic cycle. Cell *121*, 553–565.

Bertolaet, B. L., Clarke, D. J., Wolff, M., Watson, M. H., Henze, M., Divita, G., and Reed, S. I. (2001a). UBA domains mediate protein-protein interactions between two DNA damage–inducible proteins. J. Mol. Biol. *313*, 955–963.

Bertolaet, B. L., Clarke, D. J., Wolff, M., Watson, M. H., Henze, M., Divita, G., and Reed, S. I. (2001b). UBA domains of DNA damage-inducible proteins interact with ubiquitin. Nat. Struct. Mol. Biol. *8*, 417–422.

Biggins, S., Ivanovska, I., and Rose, R. D. (1996). Yeast ubiquitin-like genes are involved in duplication of the microtubule organizing center. J. Cell Biol. *133*, 1331–1346.

Chen, L., and Madura, K. (2002). Rad23 promotes the targeting of proteolytic substrates to the proteasome. Mol. Cell. Biol. *22*, 4902–4913.

Cheng, I. H., Roberts, L. A., and Tye, B. K. (2002). Mcm3 is polyubiquitinated during mitosis before establishment of the pre-replication complex. J. Biol. Chem. *277*, 41706–41714.

Ciechanover, A., Finley, D., and Varshavsky, A. (1984). Ubiquitin dependence of selective protein degradation demonstrated in the mammalian cell cycle mutant ts85. Cell *37*, 57–66.

Deshaies, R. J. (1999). SCF and Cullin/Ring H2-based ubiquitin ligases. Annu. Rev. Cell Dev. Biol. *15*, 435–467.

Deveraux, Q., Ustrell, V., Pickart, C. M., and Rechsteiner, M. (1994). A 26S protease subunit that binds ubiquitin conjugates. J. Biol. Chem. *269*, 7059–7061.

Elsasser, S., and Finley, D. (2005). Delivery of ubiquitinated substrates to protein-unfolding machines. Nat. Cell Biol. *7*, 742–749.

Elsasser, S., Chandler-Militello, D., Muller, B., Hanna, J., and Finley, D. (2004). Rad23 and Rpn10 serve as alternative ubiquitin receptors for the proteasome. J. Biol. Chem. *279*, 26817–26822.

Elsasser, S., Gali, R. R., Schwickart, M., Larsen, C. N., Leggett, D. S., Muller, B., Feng, M. T., Tubing, F., Dittmar, G. A., and Finley, D. (2002). Proteasome subunit Rpn1 binds ubiquitin-like protein domains. Nat. Cell Biol. *4*, 725–730.

Finley, D., Ciechanover, A., and Varshavsky, A. (1984). Thermolability of ubiquitin-activating enzyme from the mammalian cell cycle mutant ts85. Cell *37*, 43–55.

Funakoshi, M., Sasaki, T., Nishimoto, T., and Kobayashi, H. (2002). Budding yeast Dsk2p is a polyubiquitin-binding protein that can interact with the proteasome. Proc. Natl. Acad. Sci. USA *99*, 745–750.

Gandre, S., and Kahana, C. (2002). Degradation of ornithine decarboxylase in *Saccharomyces cerevisiae* is ubiquitin independent. Biochem. Biophys. Res. Commun. *293*, 139–144.

Glickman, M. H., Rubin, D. M., Fried, V. A., and Finley, D. (1998). The regulatory particle of the *Saccharomyces cerevisiae* proteasome. Mol. Cell. Biol. *18*, 3149–3162.

Guthrie, C., and Fink, G. R. (1991). Methods in Enzymology, Volume 194. Guide to Yeast Genetics and Molecular Biology, New York: Academic Press.

Haas, A. L., and Siepmann, T. J. (1997). Pathways of ubiquitin conjugation. FASEB J. *11*, 1257–1268.

Haracska, L., and Udvardy, A. (1995). Cloning and sequencing a non-ATPase subunit of the regulatory complex of the *Drosophila* 26S protease. Eur. J. Biochem. *231*, 720–725.

Hicke, L., Schubert, H. L., and Hill, C. P. (2005). Ubiquitin-binding domains. Nat. Rev. Mol. Cell Biol. *6*, 610–621.

Hofmann, K., and Bucher, P. (1996). The UBA domain: a sequence motif present in multiple enzyme classes of the ubiquitin pathway. Trends Biochem. Sci. *21*, 172–173.

Johnson, E. S., Ma, P. C., Ota, I. M., and Varshavsky, A. (1995). A proteolytic pathway that recognizes ubiquitin as a degradation signal. J. Biol. Chem. *270*, 17442–17456.

Kang, Y., Vossler, R. A., Diaz-Martinez, L. A., Winter, N. S., Clarke, D. J., and Walters, K. J. (2006). UBL/UBA ubiquitin receptor proteins bind a common tetraubiquitin chain. J. Mol. Biol. *356*, 1027–1035.

Kim, I., Mi, K., and Rao, H. (2004). Multiple interactions of Rad23 suggest a mechanism for ubiquitylated substrate delivery important in proteolysis. Mol. Biol. Cell *15*, 3357–3365.

Kleijnen, M. F., Shih, A. H., Zhou, P., Kumar, S., Soccio, R. E., Kedersha, N. L., Gill, G., and Howley, P. M. (2000). The hPLIC proteins may provide a link between the ubiquitination machinery and the proteasome. Mol. Cell *6*, 409–419.

Kulka, R. G., Raboy, B., Schuster, R., Parag, H. A., Diamond, G., Ciechanover, A., and Marcus, M. (1988). A Chinese hamster cell cycle mutant arrested at G2 phase has a temperature-sensitive ubiquitin-activating enzyme, E1. J. Biol. Chem. *263*, 15726–15731.

Lambertson, D., Chen, L., and Madura, K. (1999). Pleiotropic defects caused by loss of the proteasome-interacting factors Rad23 and Rpn10 of *Saccharomyces cerevisiae*. Genetics *153*, 69–79.

Lambertson, D., Chen, L., and Madura, K. (2003). Investigating the importance of proteasome-interaction for Rad23 function. Curr. Genet. *42*, 199–208. Epub 2002 Dec 13.

Lowe, E. D., Hasan, N., Trempe, J. F., Fonso, L., Noble, M. E., Endicott, J. A., Johnson, L. N., and Brown, N. R. (2006). Structures of the Dsk2 UBL and UBA domains and their complex. Acta Crystallogr. D Biol. Crystallogr. *62*, 177–188.

Madura, K. (2004). Rad23 and Rpn 10, perennial wallflowers join the melee. Trends Biochem. Sci. *29*, 637–640.

McGrath, J. P. (1991). Molecular genetic analysis of the ubiquitin-protein ligase system of *Saccharomyces cerevisiae*. Ph.D. Dissertation. Cambridge, MA: Massachusetts Institute of Technology Press.

McGrath, J. P., Jentsch, S., and Varshavsky, A. (1991). UBA 1, an essential yeast gene encoding ubiquitin-activating enzyme. EMBO J. *10*, 227–236.

Ortolan, T. G., Tongaonkar, P., Lambertson, D., Chen, L., Schauber, C., and Madura, K. (2000). The DNA repair protein Rad23 is a negative regulator of multi-ubiquitin chain assembly. Nat. Cell Biol. *2*, 601–608.

Peng, J., Schwartz, D., Elias, J. E., Thoreen, C. C., Cheng, D., Marsischky, G., Roelofs, J., Finley, D., and Gygi, S. P. (2003). A proteomics approach to understanding protein ubiquitination. Nat. Biotechnol. *21*, 921–926.

Pickart, C. M., and Cohen, R. E. (2004). Proteasomes and their kin: proteasomes in the machine age. Nat. Rev. Mol. Cell Biol. *5*, 177–187.

Raasi, S., Orlov, I., Fleming, K. G., and Pickart, C. M. (2004). Binding of polyubiquitin chains to ubiquitin-associated (UBA) domains of hHR23A. J. Mol. Biol. *341*, 1367–1379.

Raasi, S., and Pickart, C. M. (2003). Rad23 ubiquitin-associated domains (UBA) inhibit 26S proteasome-catalyzed proteolysis by sequestering lysine 48-linked polyubiquitin chains. J. Biol. Chem. *278*, 8951–8959.

Rao, H., and Sastry, A. (2002). Recognition of specific ubiquitin conjugates is important for the proteolytic functions of the UBA domain proteins Dsk2 and Rad23. J. Biol. Chem. *277*, 11691–11695.

Richly, H., Rape, M., Braun, S., Rumpf, S., Hoege, C., and Jentsch, S. (2005). A series of ubiquitin binding factors connects CDC48/p97 to substrate multiubiquitylation and proteasomal targeting. Cell *120*, 73–84.

Ryu, K. S., Lee, K. J., Bae, S. H., Kim, B. K., Kim, K. A., and Choi, B. S. (2003). Binding surface mapping of intra- and interdomain interactions among hHR23B, ubiquitin, and polyubiquitin binding site 2 of S5a. J. Biol. Chem. *278*, 36621–36627.

Saeki, Y., Isono, E., and Toh-e, A. (2005). Preparation of ubiquitinated substrates by the PY motif-insertion method for monitoring 26S proteasome activity. Methods Enzymol. *399*, 215–227.

Saeki, Y., Saito, N., Toh-e, A., and Yokosawa, H. (2002a). Ubiquitin-like proteins and Rpn10 play cooperative roles in ubiquitin-dependent proteolysis. Biochem. Biophys. Res. Commun. *293*, 986–992.

Saeki, Y., Sone, T., Toh-e, A., and Yokosawa, H. (2002b). Identification of ubiquitin-like protein-binding subunits of the 26S proteasome. Biochem. Biophys. Res. Commun. *296*, 813–819.

Salvat, C., Acquaviva, C., Scheffner, M., Robbins, I., Piechaczyk, M., and Jariel-Encontre, I. (2000). Molecular characterization of the thermosensitive E1 ubiquitin-activating enzyme cell mutant A31N-ts20. Requirements upon different levels of E1 for the ubiquitination/degradation of the various protein substrates in vivo. Eur. J. Biochem. *267*, 3712–3722.

Schauber, C., Chen, L., Tongaonkar, P., Vega, I., Lambertson, D., Potts, W., and Madura, K. (1998). Rad23 links DNA repair to the ubiquitin/proteasome pathway. Nature *391*, 715–718.

Schuberth, C., Richly, H., Rumpf, S., and Buchberger, A. (2004). Shp1 and Ubx2 are adaptors of Cdc48 involved in ubiquitin-dependent protein degradation. EMBO Rep. *5*, 818–824.

Shimada, K., Pasero, P., and Gasser, S. M. (2002). ORC and the intra-S-phase checkpoint: a threshold regulates Rad53p activation in S phase. Genes Dev. *16*, 3236–3252.

Swanson, R., and Hochstrasser, M. (2000). A viable ubiquitin-activating enzyme mutant for evaluating ubiquitin system function in *Saccharomyces cerevisiae*. FEBS Lett. *447*, 193–198.

Swanson, R., Locher, M., and Hochstrasser, M. (2001). A conserved ubiquitin ligase of the nuclear envelope/endoplasmic reticulum that functions in both ER-associated and Mat-alpha2 repressor degradation. Genes Dev. *15*, 2660–2674.

van Nocker, S., Sadis, S., Rubin, D. M., Glickman, M., Fu, H., Coux, O., Wefes, I., Finley, D., and Vierstra, R. D. (1996). The multiubiquitin-chain-binding protein Mcb1 is a component of the 26S proteasome in *Saccharomyces cerevisiae* and plays a nonessential, substrate-specific role in protein turnover. Mol. Cell. Biol. *16*, 6020–6028.

van Nocker, S., and Vierstra, R. D. (1993). Multiubiquitin chains linked through lysine 48 are abundant *in vivo* and are competent intermediates in the ubiquitin proteolytic pathway. J. Biol. Chem. *268*, 24766–24773.

Verma, R., Aravind, L., Oania, R., McDonald, W. H., Yates, J. R., Koonin, E. V., and Deshaies, R. J. (2002). Role of Rpn11 metalloprotease in deubiquitination and degradation by the 26S proteasome. Science *298*, 611–615.

Verma, R., Chen, S., Feldman, R., Schieltz, D., Yates, J., Dohmen, J., and Deshaies, R. J. (2000). Proteasomal proteomics: identification of nucleotide-sensitive proteasome-interacting proteins by mass spectrometric analysis of affinity-purified proteasomes. Mol. Biol. Cell *11*, 3425–3439.

Verma, R., Chi, Y., and Deshaies, R. J. (1997). Ubiquitination of cell cycle regulatory proteins in yeast extract. Methods Enzymol. *283*, 366–376.

Verma, R., Oania, R., Graumann, J., and Deshaies, R. J. (2004). Multiubiquitin chain receptors define a layer of substrate selectivity in the ubiquitin-proteasome system. Cell *118*, 99–110.

Walters, K. J., Kleijnen, M. F., Goh, A. M., Wagner, G., and Howley, P. M. (2002). Structural studies of the interaction between ubiquitin family proteins and proteasome subunit S5a. Biochemistry *41*, 1767–1777.

Walters, K. J., Lech, P. J., Goh, A. M., Wang, Q., and Howley, P. M. (2003). DNA-repair protein hHR23a alters its protein structure upon binding proteasomal subunit S5a. Proc. Natl. Acad. Sci. USA *100*, 12694–12699.

Watkins, J. F., Sung, P., Prakash, L., and Prakash, S. (1993). The *Saccharomyces cerevisiae* DNA repair gene RAD23 encodes a nuclear protein containing a ubiquitin-like domain required for biological function. Mol. Cell. Biol. *13*, 7757–7765.

Wilkinson, C.R.M., Seeger, M., Hartmann-Petersen, R., Stone, M., Wallace, M., Semple, C., and Gordon, C. (2001). Proteins containing the UBA domain are able to bind multi-ubiquitin chains. Nat. Cell Biol. *3*, 939–943.